Steroidal Sapogenins. XVI. Hydrolysis of 5α-22a-Spirostane Glycosides by Fungal Saponases[1,2]

By Merle M. Krider, Theone C. Cordon and Monroe E. Wall

Certain microörganisms, especially those of the genera *Aspergillus* and *Penicillium*, when grown in a medium containing steroidal saponins with 5α,22a-spirostane aglycones adaptatively produce enzymes which cleave the saponins to sapogenins. The sapogenins thus obtained are identical with those secured as a result of cleavage of saponases from higher plants or by hydrochloric acid.

In a preceding paper[3] we reported that saponins with 5α,22a-spirostane aglycones can be split by saponases from higher plants into the component steroids and sugars. The present communication deals with the hydrolysis of similar steroidal glycosides by fungal saponases.

Stoll and co-workers[4] have shown that enzyme preparations from numerous fungi can cleave the carbohydrate–steroid linkage of certain cardiac glycosides. Similar enzyme preparations had no effect on 5α,22a-spirostane glycosides. We noted, however, that a purified saponin substrate contaminated by an actively growing mold was cleaved to the steroidal sapogenin. As a result of this discovery a large number of fungi were screened for their ability to grow in and to hydrolyze steroidal saponin substrates. The data are too voluminous to tabulate, but pertinent experimental results are presented in this paper.

Of the various fungi tested, several, but not all, species of *Aspergillus* and *Penicillium* gave the best results. In some instances, moreover, different strains of the same species gave opposite results, one strain being active and the other inactive. Species of these genera and a few others which hydrolyzed significant amounts of saponin are presented in Table I. A number of genera, species of which have failed to hydrolyze 5α,22a-spirostane glycosides, are as follows: *Absidia*, *Botrytis*, *Chaectomium*, *Circinella*, *Cunninghamella*, *Gliocladium*, *Helicostylum*, *Monilia*, *Mucor*, *Myrothecium*, *Oid-*

(1) Paper XV, M. E. Wall, *et al.*, AIC 367, in press.

(2) Presented in part at the Philadelphia Meeting-in-Miniature of the American Chemical Society, January 29, 1953. Article not copyrighted.

(3) M. M. Krider and M. E. Wall, This Journal, 76, 2938 (1954).

(4) A. Stoll, J. Renz and A. Brack, *Helv. Chim. Acta*, 34, 397 (1951).

ium, Paecilomyces, Rhizopus, Sordaria, Spicaria, Stachybotrys, Stemphylium, Syncephalastrum and *Trichoderma.*

TABLE I

FUNGAL SPECIES WHICH CLEAVE 5α,22a-GLYCOSIDES

Aspergillus
awamori Nakazawa
chevalieri CZ43
flavus, ATTC 9807
glaucus CZ5
niger, NRRL 1292, 330; ALCA 5, TC-251-4247
niger van Tieghem, NRRL 328, 334, 3
phoenicis, NRRL 1956
wentii Wehmer
Penicillium
cyclopium Westling ERRL,[a] NRRL 942, 1888, 1899
chrysogenum NRRL 807
Species—many unidentified[b]
Cladosporium cladosporiodes
Fusarium sp.
Pulularia sp.

[a] This strain of this species was the contaminant which led to the discovery of fungal saponase. [b] Obtained from various plant collections sent to this Laboratory.

The ability of various fungi to cleave 5α,22a-spirostane glycosides was demonstrated to be completely adaptative. After one of these fungi has grown in a solution containing an appropriate saponin, a cell-free filtrate of this solution contains enzymes that will cleave saponins in a fresh saponin substrate. Fungal saponase is therefore considered to be an exo-cellular enzyme. However, when the same fungi are grown on various media containing *no saponin* the cell-free filtrates will not cleave saponin substrates. Likewise, fungi growing on a medium containing the cardiac glycoside, strophanthin, failed to produce saponin splitting enzymes. Hence, production of fungal saponase is dependent on the presence of saponin in the substrate.

Fungal saponase is formed, apparently equally well, in purified saponin substrates free from simple sugars, in similar substrates to which sugars (glucose or sucrose) have been added, or in crude aqueous plant extracts. Growth and cultural conditions have not been exhaustively studied, but in general the optimal conditions for growth in and hydrolysis of saponin substrates are a time period of 48–96 hr., continuous aeration of the media, and a *p*H of 5.5–6.5. If purified saponin substrates are used suitable nitrogen sources such as peptone or ammonium phosphate are required. Excellent results can be obtained, however, with crude aqueous saponin extracts without the addition of supplementary nutrients. Saponin hydrolysis proceeds equally well on these aqueous extracts under "sterile" or "non-sterile" conditions (Fig. 1).

The products of the action of fungal saponase on 5α,22a-spirostane glycosides invariably are the same as those obtained by acid hydrolysis. Similar results have been obtained with higher plant saponase.[3] The crude sapogenins being water-insoluble, are easily isolated from the media. Usually a considerable proportion of fungal hyphae is present as a contaminant. The sapogenins are readily separated from these solids by solvent extraction (*cf.* Experimental section).

Data on the hydrolysis of four typical purified saponin substrates by several typical *Aspergilli* and *Penicillia* are presented in Table II. It is apparent that under the conditions used the hydrolysis is never complete, 80% of the acid hydrolysis figure being the maximum. Although the four substrates had much the same sapogenin composition, consisting largely of glycosides of manogenin and hecogenin with minor proportions of tigogenin and gitogenin, the degree of hydrolysis varied both with the fungus and the plant source of the saponin. Explanations of these results must await completion of a study of the carbohydrate moieties of the various steroidal saponins. It is clear, however, that no single organism will give maximal hydrolysis of all saponin sources.

TABLE II

EFFECT OF VARIATIONS IN FUNGAL ORGANISM AND SAPONIN SOURCE ON HYDROLYSIS OF 5α,22a-SPIROSTANE GLYCOSIDES FROM *Agave*

Per cent. hydrolysis is based on acid hydrolysis = 100%

Fungus	Saponin source *A. ferox*	*A. lou-meyana*	*A. ceru-lata*	*A. avel-lanidens*
P. cyclopium Westling ERRL	70%	70%	40%	25%
A. tamarii Kita	..	80	15	15
P. chrysogenum	..	35	65	15
P. cyclopium Westling NRRL-942	..	..	80	50
A. flavus—ATCC 9807	55	..	75	45
A. wentii Wehmer	..	..	..	70

Experimental

Preparation of Saponin Substrates. A.—The preparation of partially purified saponin substrates made possible the study of the effect of certain variables upon the production of fungal saponase. Freshly ground plant leaves were extracted with boiling 95% aqueous ethanol and the residues with 85% ethanol. The alcohol was partially removed from the combined extracts by heating, and, after cooling, fat-soluble materials were removed by extracting with benzene. The aqueous solution was heated on a steam-bath, adding water as needed, until all the benzene and ethanol had evaporated. The saponins were extracted from the aqueous solution with butanol. The butanol extract was washed with water to remove sugars. Upon adding more water and distilling, the butanol was removed and a water solution of partially purified saponin remained. In most instances 1% peptone and 0.5% KH_2PO_4 were added and the substrate buffered at a selected *p*H. An aliquot was withdrawn for an acid-hydrolyzed control of the sapogenin content.

B.—When large volumes of substrate were required, a much simpler method of preparation was necessary. In several instances the preparation of the substrate consisted only in centrifuging the aqueous plant extract and in adjusting it to a definite *p*H.

Hydrolysis of Saponins by Fungal Saponase.—A saponin substrate was prepared (method A) from a sample of *Agave cerulata*. The *p*H was 5.7. A 50-ml. portion containing the equivalent of 80 mg. of sapogenin acetate was added to each of five 300-ml. erlenmeyer flasks. After autoclaving and cooling, the substrates were inoculated from actively growing pure slant cultures. Growing conditions were agitation on a reciprocal shaker at 90 three-inch strokes per min. for 96 hr. at room temperature, 25–28°. Fungal growth was stopped by autoclaving. The slurry was transferred to a 250-ml. centrifuge tube and shaken with 40 ml. of benzene containing 10% ethanol. The emulsion was broken by centrifuging at 2000 r.p.m. for 20 minutes. The solvent layer was collected in a suction flask and the extraction process repeated. The sapogenin extracts were washed six times with 50% aqueous ethanol to remove saponins.

The wash water was re-extracted with fresh benzene, this benzene washed three times and combined with the main sapogenin extract. Upon evaporation to dryness in a tared beaker the crude sapogenin was converted to an acetate and an analysis calculated from subsequent infrared spectral data. Results are presented in Table II as percentage of theoretical yield as determined by acid hydrolysis.

Frozen *Agave toumeyana* leaves, 2.25 kg., were ground and immediately extracted three times with a total of 8 l. boiling water. The extract was centrifuged, adjusted to *p*H 6.5 and poured into a 5-gal. carboy (method B). Into the mouth of the carboy was wired a rubber stopper previously fitted with a stirrer, sampling tube, air sparger and air vent tube. After autoclaving, *in toto*, and cooling to room temperature the substrate was inoculated with 0.5 l. of a 48-hr. culture of *Penicillium cyclopium* Westling (ERRL). As soon as the stirrer had created a uniform mixture a sample was withdrawn. Duplicate 100-ml. portions were immediately extracted with benzene–ethanol to determine zero-time free sapogenin content. Two other 100-ml. portions served as acid hydrolyzed controls for the experiment. Air was introduced into the mixture at the rate of 300–500 ml. per minute. Duplicate 50-ml. samples were withdrawn aseptically at regular intervals. After 147 hr., stirring and aeration were discontinued. Microscopic examination of the substrate at this time revealed no contaminating microörganisms, and although no alkali had been added, the *p*H of the substrate was still above 5.0. All yields were calculated as weight of sapogenin acetate per 100 ml. of substrate and are presented in Fig. 1 as percentage of theoretical yield as determined by acid hydrolysis.

The above experiment was repeated with another aqueous extract of the sample of *Agave toumeyana* with the exception that the extract was not sterilized and no special precautions were taken to prevent contamination of the substrate with other microörganisms. As a result of contamination with acid-forming organisms, there was a rapid drop in *p*H. Hence, sodium hydroxide was added as needed to maintain *p*H 5.5–6.5. Sampling was performed as before and results of analyses are depicted in the "non-sterile" curve of Fig. 1.

Solvent Extraction of Sapogenins from Dried Fermentation Solids.—While data of fungal saponase action were obtained more rapidly by direct extraction of the aqueous substrate, this procedure was not applicable to large scale runs. Recovery of sapogenins from dried fermentation solids was therefore studied.

At the conclusion of the first large scale fermentation of aqueous extract, the slurry was centrifuged. The solids were oven dried and ground to a fine powder in a Wiley mill. A representative 5-g. sample was weighed into a 125-ml. erlenmeyer flask and extracted, with continuous stirring, with 25 ml. of a selected solvent, heating on the steam-bath for 5 minutes. The mixture was filtered. The extraction process was repeated once each with 25 ml. and 10 ml. of the same solvent. The filtrates were combined and evaporated to dryness. After chromatography the yield of sapogenin was determined from infrared spectral data of the sapogenin acetates. Maximum yields of sapogenin were obtained with chloroform or with benzene containing 10% ethanol. Methanol, benzene and ether gave incomplete extraction.

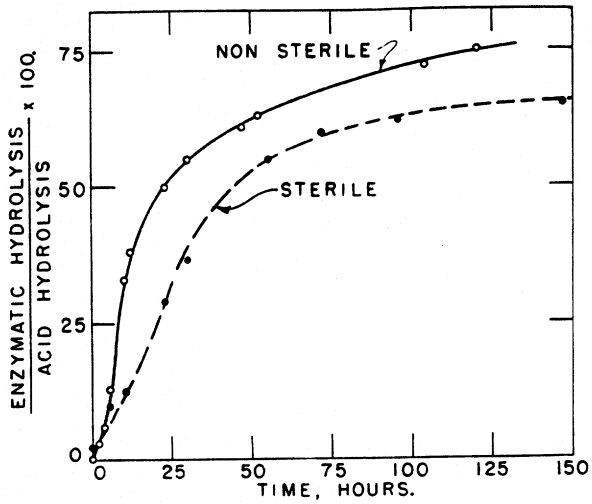

Fig. 1.—Comparison of the hydrolysis of *A. toumeyana* saponins by *P. cyclopium* when grown on "sterile" *versus* "non-sterile" extract.

Acknowledgments.—The authors wish to express their thanks to Dr. K. B. Raper, formerly of the Northern Utilization Research Branch, for identification of microörganisms; to Janet Branaman, Theodore Perlstein and George Eppley for their technical assistance; to C. Roland Eddy and Howard Jones for infrared data; and to D. S. Correll and H. S. Gentry, Section of Plant Introduction, Horticultural Crops Research Branch, Agricultural Research Service, Beltsville, Md., for the various plant samples used.